Molecular Interactions and Functionality of a Cold-Gelling Soy Protein Isolate

G.L. Cramp, P. Kwanyuen, and C.R. Daubert

ABSTRACT: A soy protein isolate (SPI) was thermally denatured at a critical concentration of 8% protein for 3 h at 95 °C, resulting in a powder that was readily reconstituted at ambient temperature and that demonstrated improved heat stability and cold-set gel functionality when compared to a control SPI. When SPI was heated at 3% protein equivalently, prior to reconstitution to 8% protein, the final viscosity was about 3 orders of magnitude less than the original sample. The viscosity of SPI heated at 3% protein was still nearly 2 orders of magnitude less than the original sample after both samples were reheated at 8% protein. These results suggested that heat denaturation at low protein concentrations limited network formation even after the protein concentration and interaction sites increased, impacting the isolate's cold gelling ability. Gelation was prevented upon treatment of SPI with iodoacetamide, which carbaminomethylated the cysteine residues, establishing the role of disulfide bonds in network formation. The viscosity of the 8% protein dispersion was also reduced by 2 orders of magnitude when treated with 8 M urea, and when combined with 10 mM DTT the gel viscosity was decreased by another order of magnitude. These results suggested that hydrophobic interactions played a primary role in gel strength after disulfide bonds form. The need for a higher concentration of protein during the heating step indicated that the critical disulfide bonds are intermolecular. Ultimately, the functionality produced by these protein–protein interactions produced a powdered soy protein isolate ingredient with consistent cold-set and thermal gelation properties.

Keywords: cold-gelling, critical concentration, disulfide bonds, hydrophobic interactions, soy protein isolate

Introduction

Soy protein isolate (SPI) is a high-quality plant protein source with associated nutritional benefits that have increased its utilization to the food industry in recent years. Soy protein is one of several heat-gelling proteins. Recently, cold-set gelation of soy protein gels has been demonstrated using a preheating step to denature the proteins, followed by addition of calcium to induce gelation through electrostatic interactions (Maltais and others 2005). This example of cold gelation demonstrated the potential for modifications to SPI to improve and expand current functional capabilities.

The soy protein subunits that aggregate and become insoluble during heating contribute to gelation (Wolf 1970; Petrucelli and Anon 1995a, 1995b; Sorgentini and others 1995). The α', α, and β subunits have been observed to aggregate and become insoluble above certain protein concentrations during heating (Utsumi and others 1984; Sorgentini and others 1995). The basic subunit has also been shown to become insoluble during heating (Wolf 1970; Utsumi and others 1984; Sorgentini and others 1995). The nature of these aggregates and a greater understanding of soy protein gels is a continuous subject for research.

The presence of disulfide bonds and sulfhydryl groups in soy protein has been previously investigated, indicating most opportunities for disulfide bonding are in the 11S fraction (Koshiyama 1971; Thanh and Shibasaki 1977; Shimada and Cheftel 1988). Sulfhydryl groups provide the opportunity for irreversible covalent interactions. The 7S fraction contains 4 sulfhydryl groups that participate in 2 intramolecular disulfide bonds, whereas 11S has 48 sulfhydryl groups, many of which interact in bonds between the acidic and basic subunits (Koshiyama 1971; Hermansson 1978).

Although most researchers agree that sulfhydryl/disulfide interchange plays a part in gelation due to the decrease in gel strength upon the addition of disulfide reducing agents, their actual role with respect to other molecular interactions has not been fully established and may depend on gelation temperature and protein concentration. Circle and others (1964) observed that soy protein gels are irreversible under heating conditions of 100 °C for 30 min and concluded that disulfide bonds must be a part of the gelation mechanism. Alternatively, Catsimpoolas and Meyer (1970) proposed a schematic where the gel formed at 80 °C was reversible and therefore must be formed through noncovalent interactions. In addition, Catsimpoolas and Meyer (1970) found that small amounts of reducing agents reduced gel strength, but large amounts allowed the gel to unfold and create more preferential interactions. However, further support for disulfide bonds was demonstrated when SPI gels were shown to increase in firmness with temperature up to 120 °C, coincident with a decrease in free sulfhydryl groups and protein solubility (Shimada and Cheftel 1988).

The objective of this study was to utilize soy protein concentration and thermal denaturation to produce a soy protein isolate that gels at ambient temperatures upon the addition of water. Insight into the functional mechanisms of cold gelation, depending on protein concentration at denaturation, is provided. An understanding of the functional mechanism that provided the versatility of this SPI ingredient enables further expansion of soy protein-based ingredients.

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Materials and Methods

Chemicals

Urea, dithiothrietol (DTT), iodoacetamide, β-mercaptoethanol (β-ME), hexane, Trizma base, sodium dodecyl sulfate (SDS),

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glycine, ethylenediaminetetraacetic acid (EDTA), bis-acrylamide (40%), glycerol, bromphenol blue, ammonium persulfate, methanol, acetic acid, and sucrose were purchased from Sigma Chemical (St. Louis, Mo., U.S.A.). All other chemicals were of analytical grade. Distilled water or purified Milli-Q water (Millipore Corp., Billerica, Mass., U.S.A.) of 18 mΩ-cm resistivity was used for the preparation of all analytical reagents and buffers.

Preparation of soy protein isolate

Soy protein isolate (SPI) was prepared from soybeans (*Glycine max* L. *Merr.,* cv. Brim). Soybean seeds were ground in a Retsch centrifugal grinder, Model ZM100 (Newtown, Pa., U.S.A.), equipped with a 24-tooth rotor and a 1.0-mm stainless steel ring using a motor speed set at 14000 rpm. This setting produced ground samples with a uniform particle size of less than 0.5 mm for efficient oil and protein extraction. Oil was extracted from the ground meal using a Soxhlet apparatus with warm hexane. Protein isolate was prepared batchwise from the defatted meal using the commercial procedure described by Lusas and Rhee (1995) (Figure 1). Due to limited capacity of our laboratory equipment, the preparation resulted in approximately 3% isolate solution, determined as described by Bradford (1976). The majority of the protein solution was lyophilized and used as a control while the remainder was modified as described herein. The lyophilized SPI was ground in a centrifugal grinder, but with a 0.5-mm sieve size, to produce SPI in a powdered form. The protein content of the lyophilized SPI was determined by the Dumas combustion method (Nielsen 1998). Unless otherwise stated, all experiments and procedures were performed at room temperature.

Protein modification

The lyophilized SPI was rehydrated to 8% protein (Figure 2). A portion of the 8% solution was lyophilized again with no further treatment, serving as the control (control SPI). The remaining 8% solution and the remaining portion of the protein still in solution at approximately 3% protein were heated for 3 h at 95 ± 3 °C and lyophilized. Both lyophilized samples were ground to powdered form as previously described and stored at 4 °C. The heated samples are referred to as 8% SPI and 3% SPI.

Sample preparation for viscoelastic tests

The 8% SPI and the control SPI were prepared to 8% protein using either distilled water, 8 M urea, 10 mM DTT, or 8 M urea + 10 mM DTT. The 3% SPI was also prepared to 8% protein in distilled

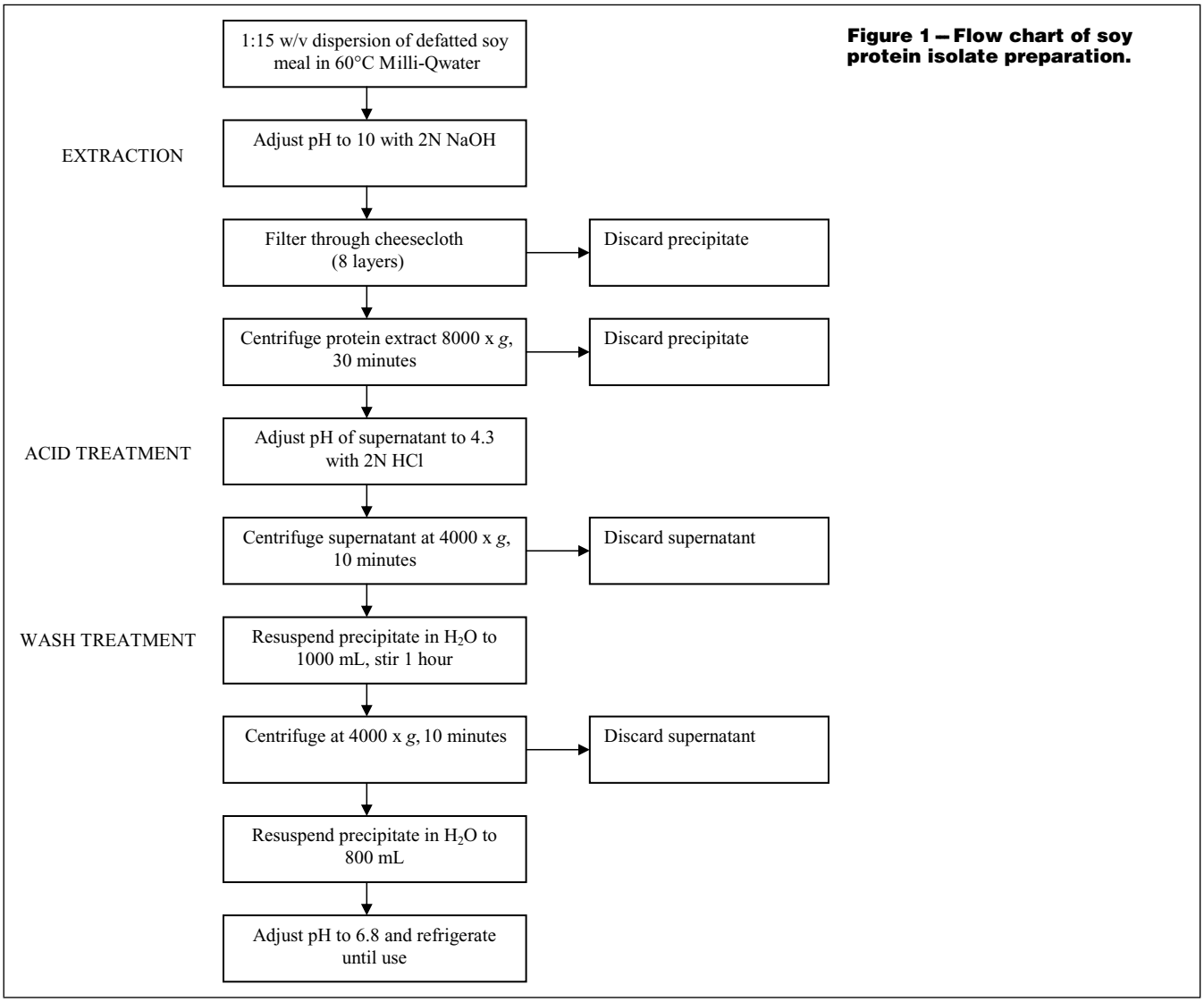

Figure 1—Flow chart of soy protein isolate preparation.

water. All samples were allowed to rehydrate overnight and tests were performed within 48 h of sample preparation.

Measurement of viscoelastic properties

A Rheologica Stresstech Rheometer (ATS Rheosystems, Bordentown, N.J., U.S.A.) was used to measure viscoelastic properties of all test solutions as a function of frequency (0.005 to 10 Hz) and temperature (from 10 to 90 °C) within a predetermined linear viscoelastic region. Thermal measurements began at room temperature (25 °C), and were continuously recorded as the samples were cooled to 10 °C and heated to 90 °C (2.5 °C/min). The sample temperature was held at 90 °C for 30 min, simulating potential processing conditions. The samples were cooled to a final temperature of 25 °C (2.5 °C/min), establishing a baseline comparison for sample temperature stability from the beginning of the test to the end. Pressure was applied to the sample using an ATS Rheosystems sealed cell to prevent moisture loss, and data were recorded in triplicate.

Polyacrylamide gel electrophoresis

The subunit profile of soluble and insoluble soy protein fractions was evaluated using a Bio-Rad (Richmond, Calif., U.S.A.) Protean II vertical slab gel apparatus according to Chua (1980) with the following modifications. A soybean reference protein (SRP) was prepared according to the procedure of Kwanyuen and Wilson (2000). The control SPI, 3% SPI, and 8% SPI samples were rehydrated to 8% protein and centrifuged at 5200 × *g* for 10 min to separate the soluble and insoluble fractions. The supernatant containing the soluble protein was pipetted into a separate container and the pellets were resuspended to 2 mL in water. Protein concentrations of the soluble and insoluble fractions were determined according to Bradford (1976). Each sample contained about 25 mg/mL protein. The 3 SPI samples and SRP were mixed at a 1:1 ratio with 0.06 M Tris-HCl, pH 8.0, 5% SDS prepared with 0.1 M β-mercaptoethanol. In some samples, 0.1 M β-mercaptoethanol was excluded in order to evaluate the disulfide banding pattern. Proteins were completely dissociated in a boiling water bath for 10 min. The tracking dye was added to the samples at a concentration of 10% glycerol and 0.025% bromophenol blue. The samples were loaded onto the gel and electrophoresed using a 10% to 20% gradient polyacrylamide gel. Blank wells were left in-between loaded samples to prevent cross-contamination during migration and to facilitate accurate quantification with densitometry. A constant current of 10 mA/gel was used until tracking dye reached the bottom of the gel.

Gels were fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for an hour on an orbit shaker and then stained overnight in 0.25% (w/w) Coomassie brilliant blue R250, 40% (v/v) methanol, and 10% (v/v) acetic acid on the orbit shaker. Gels were destained in 40% (v/v) methanol and 10% (v/v) acetic acid. The destaining solution was changed when it reached a similar color to the gel. After at least 3 changes of the destaining solution, gels were soaked in distilled water for 15 min, scanned with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, Calif., U.S.A.), and analyzed with Molecular Dynamics ImageQuant software. Volume integration was used in data analysis to determine the total absorbance of entire protein bands. The apparent absorbance of each protein subunit was obtained by subtracting the local average background absorbance from the total absorbance of the protein subunit within the same gel volume. Gels were sandwiched between cellophane and dried in a Bio-Rad GelAir dryer.

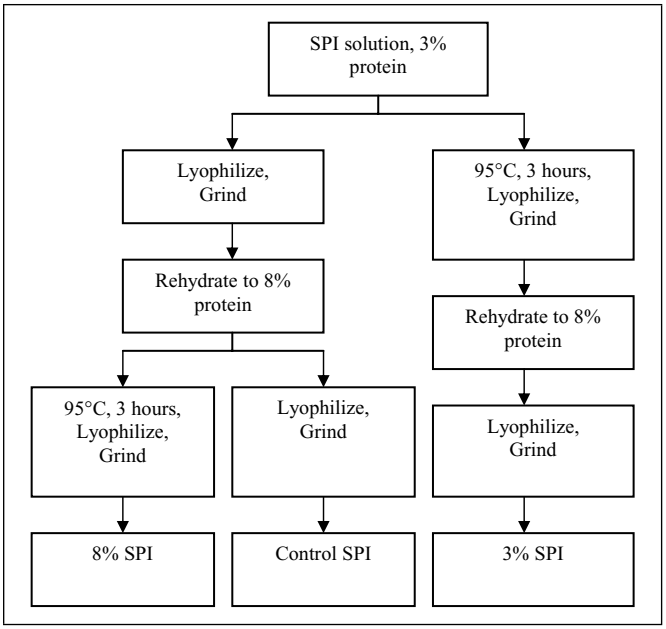

Figure 2—Preparation of soy protein isolate samples. All samples were rehydrated to 8% protein (w/w) for viscoelastic testing.

Differential scanning calorimetry

Thermal denaturation of the soy proteins was assessed with a Perkin-Elmer DSC 7 (Wellesley, Mass., U.S.A.), calibrated with indium and mercury using N_2 purge gas. Control SPI and 3% SPI were rehydrated to 20% protein and analyzed in a hermetically sealed stainless steel pan with an empty pan of similar weight as the reference. The samples were heated from 10 to 110 °C at 10 °C/min, held at 110 °C for 1 min, cooled to 10 °C at 10 °C/min, held at 10 °C for 1 min, and heated to 110 °C at 10 °C/min. Denaturation temperatures were determined at peak height and enthalpy was determined from area under the peak for the 7S and 11S protein fractions.

Determination of sulfhydryl content

Sulfhydryl content was measured according to the method of Shimada and Cheftel (1988) with modifications. Control SPI and 8% SPI were solubilized at 4 mg/mL in 0.086 M Tris-HCl at pH 8.0 containing 0.09 M glycine, 4 mM Na_2EDTA, 0.5% SDS, 6 M urea, and 10 mM dithiothreitol (DTT). The disulfide reduction was carried out at room temperature under vacuum for 1 h and excess DTT was then removed by gel filtration using Sephadex G-25 column chromatography previously equilibrated with 0.086 M Tris-HCl at pH 8.0 containing 0.09 M glycine and 4 mM EDTA. The sulfhydryl content was determined according to the procedure of Ellman (1959) using 10 mM DTNB in 0.1 M NaH_2PO_4 at pH 8.0. A Shimadzu 2101 UV-VIS spectrophotometer (Tokyo, Japan) was used to quantify the free sulfhydryl content at wavelength 412 nm.

Protein content was determined after gel filtration using the procedure of Lowry and others (1951) as modified by Bensadoun and Weinstein (1976), a procedure involving precipitation of the protein from solution to remove interfering chemicals prior to Lowry analysis. The protein solution was sampled at 0.2, 0.4, 0.6, 0.8 and 1.0 mL and diluted in 3 mL of water. Twenty-five microliters of sodium deoxycholate were added to each protein sample and reacted for 15 min. The protein was precipitated by addition of 1 mL of 24% trichloroacetic acid and collected upon centrifugation at 3300 × *g* for 30 min. The protein pellets were redissolved in 1.5 mL Lowry reagent C (50 parts of reagent A [2% Na_2CO_3 in 0.10 N NaOH] + 1 part of reagent B [0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate]). After 10 min of incubation, 0.15 mL of 1 N Folin–Ciocalteu reagent was added, and the solutions reacted for 45 min in the dark. Bovine

serum albumin was used to generate a standard curve. Absorbance was measured on the Shimadzu spectrophotometer at wavelength 730 nm.

Carbaminomethylation of soy protein isolate

Control SPI (lyophilized only once after isolation) was reacted with iodoacetamide according to the method of Aitken and Learmonth (1996). Protein solutions (8%) were dissolved in 0.6 M Tris-HCl, pH 8.6 containing 8 M urea. Disulfide bonds were reduced by adding 5 M DTT at 0.166% of the total weight. The protein solution was sealed, evacuated, and flushed with nitrogen and held for 3 h to fully reduce the disulfide bonds while preventing air oxidation of sulfhydryl groups to disulfide bonds. The sulfhydryl groups were then covalently linked to iodoacetamide, which was added as 10% of the total weight, using a concentration of 500 mM. Carbaminomethylation proceeded in darkness at 38 °C for 30 min under anaerobic conditions, as described previously. The solution was dialyzed against water for 40 h with 3 changes of water. The protein sample was then lyophilized and ground to a powder in the same manner as previously described. The protein content was determined to be 98.2% by the Dumas combustion method (Nielsen 1998). The sample was prepared at 8% protein, heated for 3 h at 95 ± 3 °C, lyophilized, and then ground to a powder in the same manner as previously described. A control was prepared without a thermal treatment. The powders were rehydrated to 8% protein and visual observations were recorded.

Results and Discussion

Viscoelastic response in water and urea

A critical concentration for heat induced gelation of soy proteins was previously reported to be approximately 8% SPI powder (Circle and others 1964; Catsimpoolas and Meyer 1970; Sorgentini and others 1995). During laboratory isolation, the soy protein concentration was around 3%. In this study, that soy protein solution was freeze dried and rehydrated at 8% protein before thermally inducing gelation, then dried to a powder (Figure 2). In a commercial setting, proteins may become completely denatured during isolation (Hermansson 1978; Wagner and others 1992). Commercially prepared soy protein isolates have been shown to provide different functionality and mechanisms of gelation depending on degree of denaturation, method of processing, or other unknown causes (Hermansson 1978, 1986; Chronakis 1996). Wagner and others (1992) suggested that the denatured soy protein formed insoluble aggregates that would not interact in new structures. Although the protein concentration at denaturation of fully denatured commercial isolates is not known, this study was also conducted at 3% protein based on laboratory data to determine the functionality of a protein already subjected to heat denaturation at low concentration. The SPI powders were determined to be 87.6% protein by the Dumas combustion method (Nielsen 1998). Although thermal treatments occurred at different protein concentrations, used to label the samples, all samples were on an equivalent 8% protein basis for testing.

The complex viscosity (η^*) of the control SPI, the 3% SPI, and the 8% SPI is shown in Figure 3A. The control and 3% SPI displayed similar viscosities through varying oscillatory speeds. After heating, the control SPI, 3% SPI, and 8% SPI all demonstrate the elastic properties of a gel, but the 3% SPI never achieved the strength of the gels initially heated at 8% protein (Figure 3B). The η^* for the 8% SPI was higher than the 3% SPI and control SPI at low frequencies, but decreased in viscosity at higher frequencies. The 3% SPI and control SPI did not display the same viscosity behavior as the 8% SPI because these isolates already exhibited a lower viscosity, indicating fewer protein interactions.

The viscoelastic response of the control SPI and 8% SPI with respect to temperature is shown in Figure 4A and 4B. The 8% SPI demonstrated cold-gelling ability immediately upon hydration at 25 °C and stability throughout heating and cooling (Figure 4B), whereas the control SPI hydrated at the same protein content did not achieve this functionality until it was heated to 90 °C for 30 min (Figure 4A). The 3% SPI never reached the same level of η^* as the control SPI and 8% SPI (data not shown). The stability of the 8% SPI viscosity through a heating-cooling cycle demonstrated elastic behavior typical of a gel.

Previous insight into soy protein isolate structure involved various chemical reagents: cysteine (Circle and others 1964; Wang and Damodaran 1990), β-mercaptoethanol (β-ME) (Briggs and Wolf 1957; Catsimpoolas and Meyer 1970; Utsumi and others 1984; Wolf 1993), dithiothreitol (DTT) (Wolf 1993; McKlem 2002), N-ethylmaleimide (NEM) (Briggs and Wolf 1957; Catsimpoolas and Meyer 1970; Shimada and Cheftel 1988; Wang and Damodaran 1990), iodoacetamide (Wolf 1993), sodium borohydride ($NaBH_4$) (Wolf 1993), sodium thiocyanate (NaSCN) (Nagano and others 1994), sodium sulfate (Na_2SO_3) (Petrucelli and Anon 1995b), urea (Catsimpoolas and others 1969; Petrucelli and Anon 1995b), sodium dodecyl sulfate (SDS) (Petrucelli and Anon 1995b), and guanidine hydrochloride (GHCl) (Nagano and others 1994). Use of these reagents revealed the existence of disulfide bond formation/interchange, hydrogen bonds and electrostatic and hydrophobic interactions in thermally induced soy protein gels as mechanisms for soy protein gelation.

Urea has previously shown interference with SPI gelation, demonstrating the presence of hydrophobic interactions in traditional SPI gelation (Catsimpoolas and others 1969; Catsimpoolas

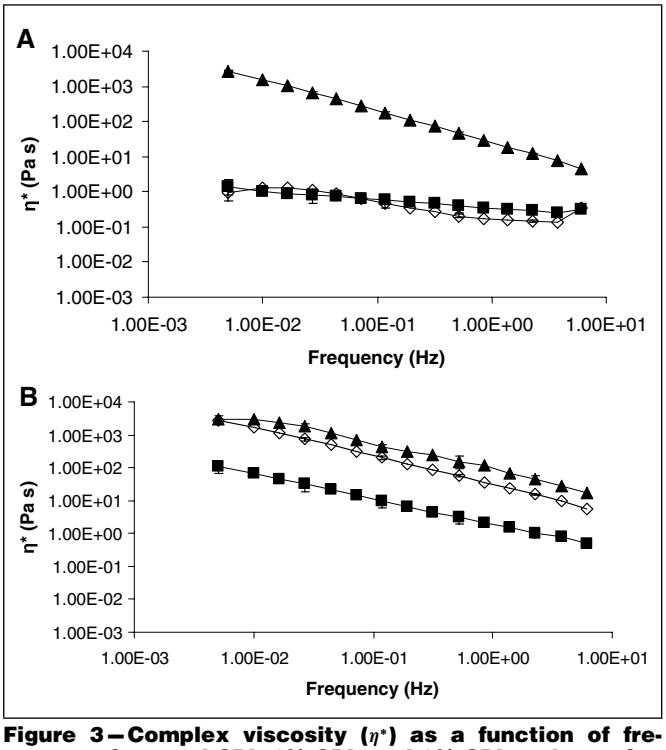

Figure 3—Complex viscosity (η^*) as a function of frequency of control SPI, 3% SPI, and 8% SPI to determine concentration effects. Control SPI (◇), 3% SPI (■), and 8% SPI (▲) rehydrated at 8% protein (w/w) (A) at 25 °C. (B) At 25 °C after heating to 90 °C. Error bars represent 1 standard deviation of 3 observations.

and Meyer 1970; McKlem 2002). Hydrophobic interactions have been shown to be important at lower gelation temperatures (80 °C), where only the sulfur deficient 7S group is denatured (Catsimpoolas and Meyer 1970). In this study, urea prevented interactions within the control SPI, and a viscosity increase did not occur during heating (Figure 4A). The response of the control SPI upon the addition of urea indicated that hydrophobic interactions were primarily responsible for the viscosity increase. Urea interrupted the hydrophobic interactions in the 8% SPI to the viscosity level of the control SPI, demonstrating the contribution of hydrophobic interactions to the cold-gelling SPI ingredient (Figure 4A and 4B).

The importance of hydrogen bonding was supported by the presence of thermally reversible activity for the control and 8% SPI. The increase in viscosity when cooled to 10 or 25 °C and the decrease in viscosity when heated to 90 °C, both with and without urea, supported the notion that hydrogen bonding increased the final viscosity of the cooled product. Hydrogen bonds were previously reported to be involved in soy protein gel formation (Utsumi and Kinsella 1985; Nagano and others 1994; McKlem 2002).

While the data support hydrophobic interactions as a primary interaction responsible for soy protein gel strength, a study by Sorgentini and others (1995) indicated that after heating at low concentrations, the protein remained denatured in the soluble state with hydrophobic sites exposed. The insoluble state at low concentrations had a low surface hydrophobicity, indicating that hydrophobic interactions led to aggregation and insolubility. Therefore, after heating at a concentration of approximately 3% protein, the denatured soluble protein should be able to interact when the protein is rehydrated at a higher concentration, aggregate, and become insoluble. However, Figure 3 reveals that rehydrating protein above the critical concentration that was heated below the critical protein concentration will not produce the same viscosity increase as protein denatured above the critical concentration. Even when the 3% SPI was reheated at the higher concentration, it did not reach the viscosity of protein solutions originally heated at the higher concentration. The inability to reach a higher viscosity suggests irreversible denaturation of the protein, which prevents the formation of more functional interactions under more favorable conditions, that is, higher concentration, even if the hydrophobic groups are exposed as described by Sorgentini and others (1995). This idea is supported by Wagner and others (1992), who found that soy protein isolates denatured during commercial isolation formed insoluble aggregates that would not interact in new structures.

Denaturation of the proteins determined with differential scanning calorimetry

Differential scanning calorimetry (DSC) was employed to determine the extent of protein denaturation of control SPI and 3% SPI (Figure 5) to support the assumption of irreversible denaturation. The control SPI shows a peak where 11S is denatured around 95 °C. This peak disappears when the control SPI is cooled and heated a 2nd time, providing a baseline for SPI denaturation. The 3% SPI was fully denatured during the 1st heating curve, demonstrating that protein heated below critical concentration denatures, potentially exposing hydrophobic sites as indicated in the study of Sorgentini and others (1995). The lower viscosity of the 3% SPI in comparison to the 8% SPI, even after reheating at a higher concentration, further supports the idea that 3% SPI denatures when heated, irreversibly aggregating into a conformation less favorable to gelation mechanisms. The irreversible behavior of 3% SPI suggests that while hydrophobic interactions are important, covalent interactions that form during denaturation produce the protein functionality. Otherwise, the 3% SPI would form stronger interactions with exposed hydrophobic groups upon reheating. The idea that hydrophobic interactions do not produce gel strength alone is further supported by Wang and Damodaran (1990), who state that a self-supporting protein gel with thermal and mechanical stability is dependent on entanglements. The entanglements are dependent on protein concentration and molecular weight. In this event, the true role of disulfide bond formation was to increase the molecular weight of the proteins above a critical molecular weight, enabling gelation (Wang and Damodaran 1990). The prerequisite of a critical molecular weight may deem intermolecular disulfide bond formation essential to soy protein gelation. The idea that disulfide bonds are essential to soy protein gelation has been supported by others

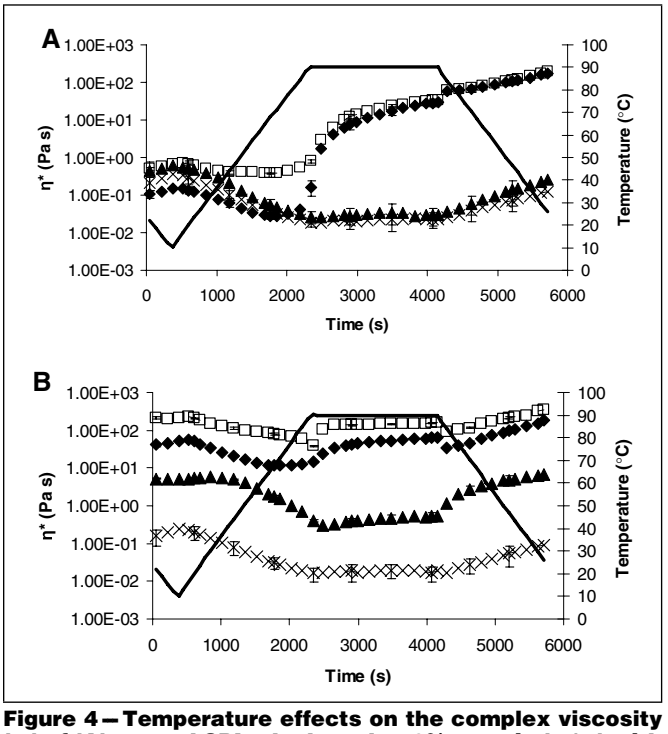

Figure 4—Temperature effects on the complex viscosity (η^*) of (A) control SPI rehydrated at 8% protein (w/w) with various denaturants. (B) 8% SPI rehydrated at 8% protein (w/w) with various denaturants. Control SPI and 8% SPI in water (□), w/ urea (▲), w/ DTT (♦), and w/ urea+DTT (x). Temperature changes are also recorded (---). Error bars represent 1 standard deviation of 3 observations.

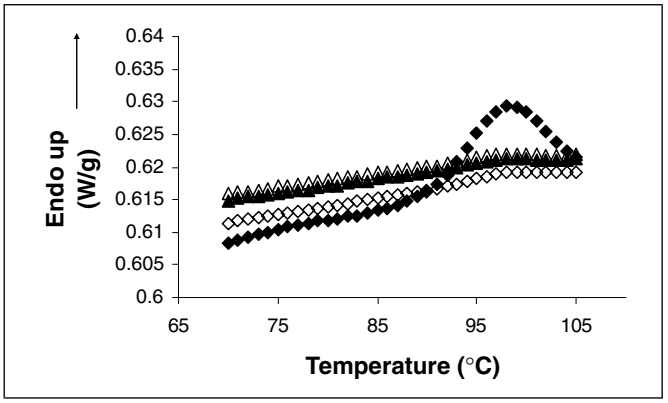

Figure 5—DSC thermogram showing heating curves for control and 3% SPI. Each sample shows a 1st and 2nd heating curve. All but control 1 are fully denatured. Control 1 (♦), 3% heated 1 (▲), control 2 (◊), 3% heated 2 (Δ).

by the observation of the loss of gelation with the addition of reducing agents (Circle and others 1964; Shimada and Cheftel 1988; Petrucelli and Anon 1995b).

Viscoelastic response in modifying agents

The role of disulfide bond formation in the control SPI and 8% SPI was further investigated with the reducing agent DTT. The DTT reduced the viscosity of both the control SPI and 8% SPI (Figure 4A and 4B), but this effect was seen more in the control SPI (Figure 4A). The presence of DTT diminished the η^* of the control SPI 10-fold upon initial rehydration at 25 °C and cooling to 10 °C. This η^* reduction may be due to breakage of disulfide bonds present in the control SPI, indicating the importance of these bonds initially. It would appear, as proposed by McKlem (2002), that disulfides are not important after heating to 90 °C, when hydrophobic interactions become important, as the control SPI containing DTT increased in viscosity with heating to the same point as the control SPI without DTT. However, the optimum temperature for DTT reaction is around 20 °C at a neutral pH and the half-life of DTT decreased nearly 10-fold with every 20 °C increase in temperature (Stevens and others 1983). Therefore, DTT likely maintained disulfide reduction of the protein initially but decreased in stability as temperature increased to 90 °C, allowing disulfide bonds to form again.

When urea and DTT were used in combination and added to the 8% SPI solutions, the cumulative decrease in viscosity was 10 times greater than with urea alone (Figure 4B). The viscosity was relatively similar for both control SPI and 8% SPI when urea and DTT were used jointly (Figure 4A and 4B), indicating that DTT reduced disulfide bonds that were initially in the protein but inaccessible until urea was present to denature the protein and permit DTT access to internal bonds. When the DTT was deactivated upon heating, the urea likely prevented protein interactions that would allow new disulfide bonds to form.

Disulfide/sulfhydryl interchange during heating would create intermolecular bonds between the proteins from the intramolecular disulfide bonds and free sulfhydryl groups currently present. Disulfide/sulfhydryl interchange has been previously proposed by Shimada and Cheftel (1988) who found approximately 6 μmol sulfhydryl/g protein. In this study, the soy protein isolate was found to contain 0.20 to 0.35 μmol sulfhydryl/g protein in the reduced state, but the difference in sulfhydryl content may be attributed to cultivar differences. According to Figure 4A and 4B, the

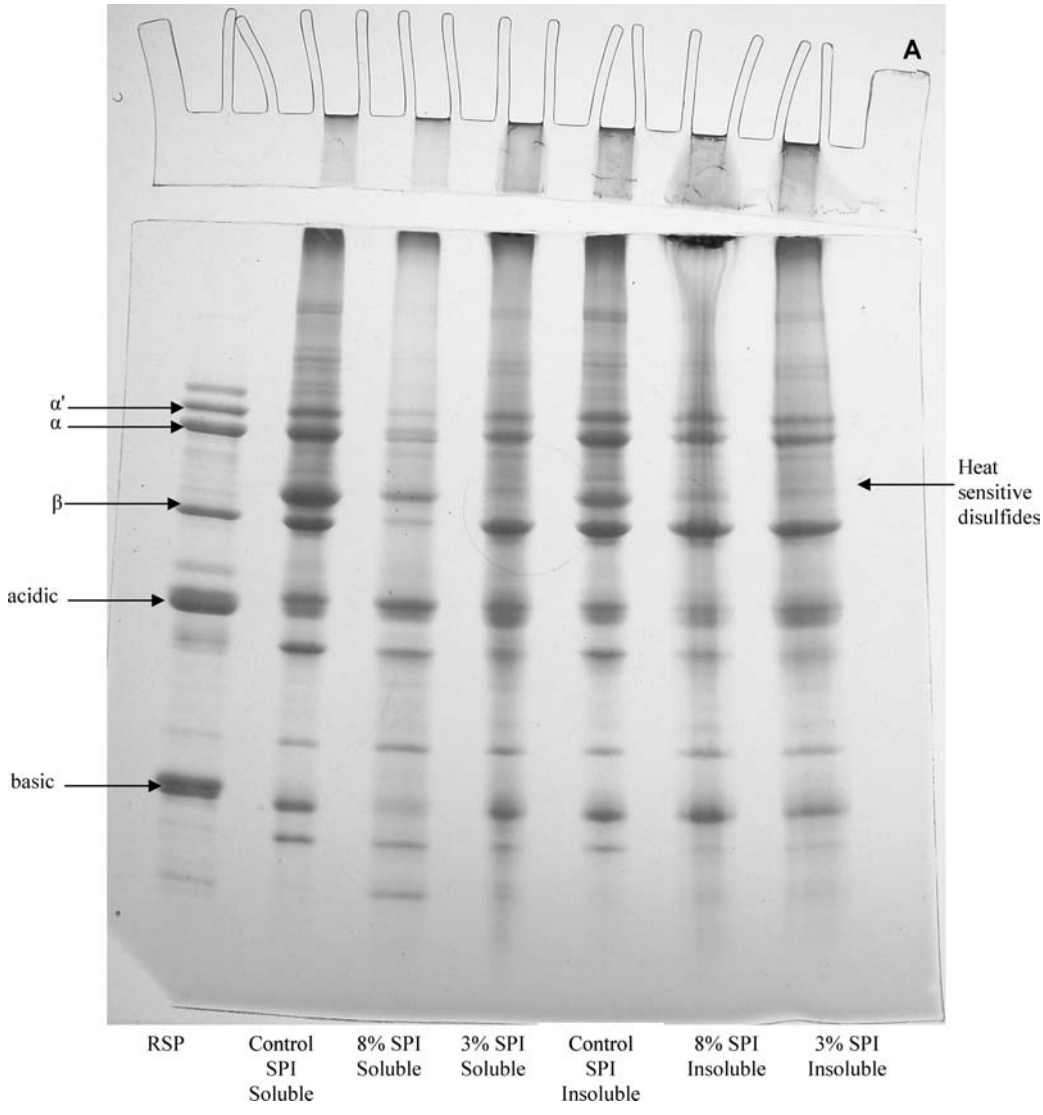

Figure 6—SDS-PAGE of soluble and insoluble fractions of Control, 8%, and 3% SPI (A) without β-ME. (B) With β-ME.

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disulfide bonds do not contribute as much to viscosity as hydrophobic interactions, based on the viscosity difference upon the addition of urea as opposed to urea + DTT. However, intermolecular disulfide bonds may explain the critical concentration requirement. Sulfhydryl/disulfide interchange would require the heating step to occur at a concentration that permits a sufficient amount of protein interaction and the overall number of disulfide bonds would not necessarily change.

Effect of iodoacetamide, urea, and dithiothreitol in combination on gelation

While DTT primarily reduces disulfide bonds and becomes inactivated over time, especially upon exposure to high temperature, iodoacetamide covalently complexes with sulfhydryl groups, preventing disulfide formation over time. Therefore, iodoacetamide was reacted with control SPI in the presence of DTT and urea to reduce disulfide bonds and prevent reformation. The presence of urea permitted DTT access to internal disulfide bonds, and reduction to free sulfhydryl groups took place under optimum time and temperature conditions. While the sulfhydryl groups were in the reduced state, the iodoacetamide covalently bonded with them, preventing reformation of disulfide bonds. The absence of disulfide bonds produced an SPI with a viscosity similar to water, even after heat treatment for 3 h at 95 ± 3 °C. The reduced solution began to phase separate immediately without agitation, suggesting that intermolecular disulfide bonds are required for SPI gelation functionality, and other interactions are dependent on the initial presence and viscosity increase produced by disulfide bonds. The need for intermolecular disulfide bonds provides support for Wang and Damodaran (1990), who demonstrate that gelation is based on entanglements at a critical molecular weight.

Evidence of intermolecular disulfides in gelation

Soy protein gels have been considered both reversible and irreversible, largely depending on the gelation conditions, such as temperature and protein concentration, involved. The presence of intramolecular or intermolecular sulfhydryl groups may assist in explaining the temperature-dependent reversible nature of soy protein gels. The SDS-PAGE was undertaken in the presence and absence of β-ME and banding patterns were examined for the soluble and insoluble portions of the control SPI, 3% SPI, and 8% SPI. There are only small amounts of cysteine in the subunits of 7S

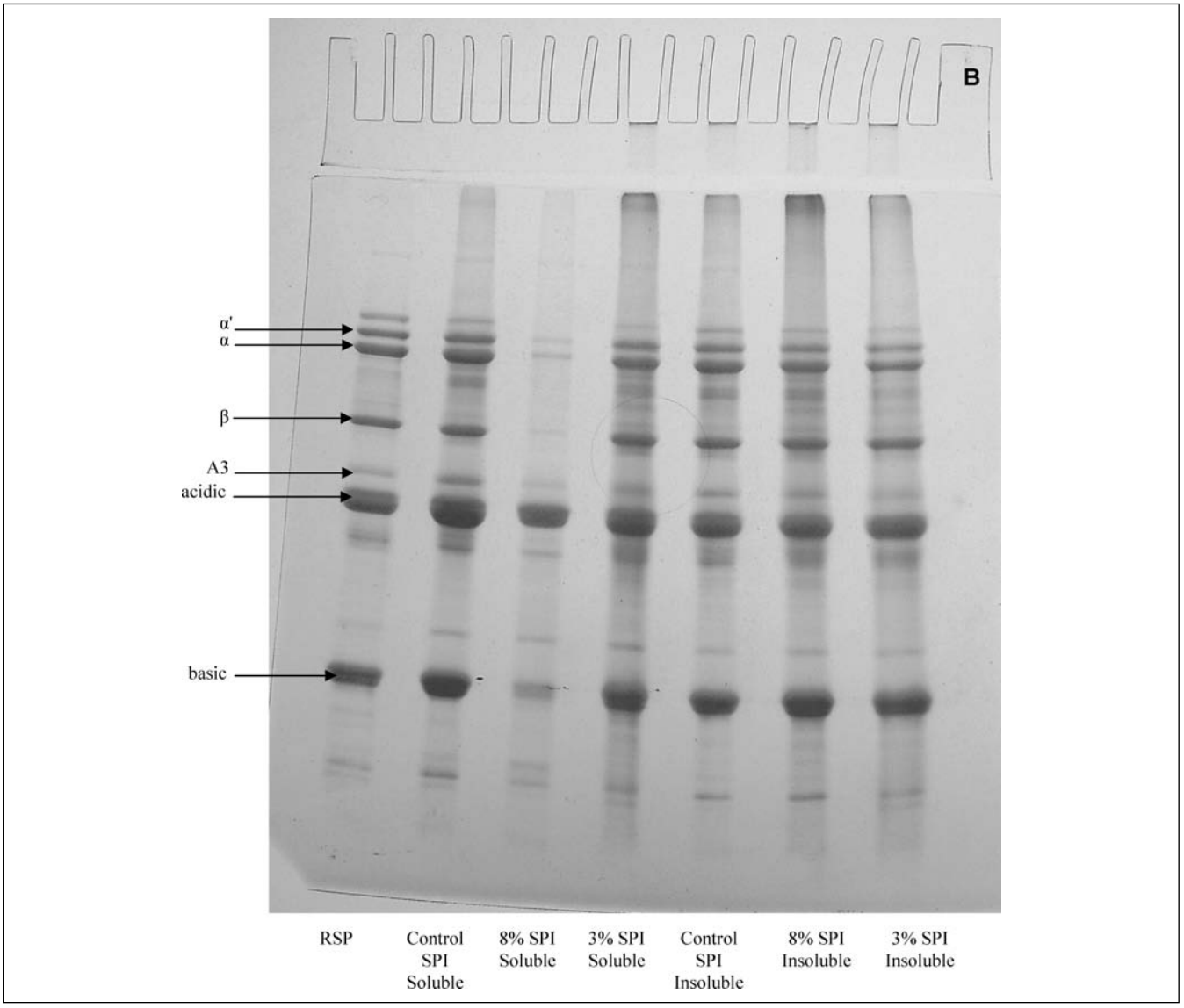

Figure 6—(Continued).

(Koshiyama 1971; Thanh and Shibasaki 1977). Therefore, the 11S would be essential for the disulfide bonding effect, while the 7S and 11S would be expected to associate noncovalently (Petruccelli and Anon 1995b).

Concentration and extent of denaturation each influence the amount of insoluble protein present in soy protein isolate, and subsequently the viscosity. The DSC results have shown that the 7S component denatured at a much lower temperature ($T_{max} = 75\ ^{\circ}C$) than 11S ($T_{max} = 95\ ^{\circ}C$) (data not shown). Earlier study has shown that these values range from 68 to 82 °C for 7S and 83 to 95 °C for 11S (German and others 1982; Sorgentini and others 1995; McKlem 2002). These differences in denaturation temperatures may be attributed to protein fraction variations between cultivars. Sorgentini and others (1995) determined that heating aqueous dispersions at concentrations greater than 8% SPI powder caused increasing amounts of protein to aggregate and become insoluble. At 80 °C, mainly 7S protein subunits are capable of denaturation and aggregation. At 100 °C, both 7S and 11S subunits may denature and subsequently aggregate. The amount of insoluble protein increased quickly, probably as a result of enhanced protein contact resulting in aggregation above 8% protein. The study of Sorgentini and others (1995) also supported Wang and Damodaran's theory that once a critical molecular weight is reached, the proteins may entangle and gel.

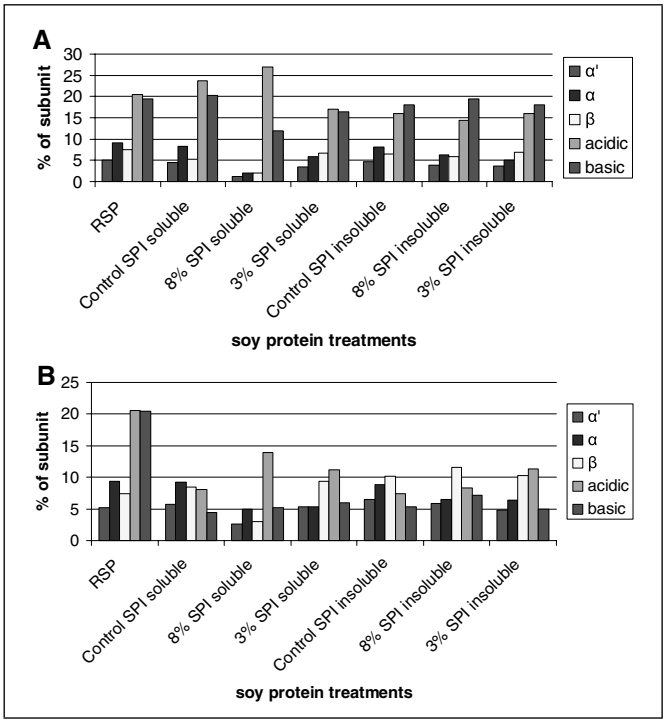

Figure 7—SDS-PAGE densitometer analysis showing percentage of each SPI subunit present in soluble and insoluble control, 8% SPI, and 3% SPI (A) with β-ME (B) without β-ME.

The SDS-PAGE electrophoresis performed in the presence and absence of β-ME was employed to determine which subunits were present in the soluble fraction compared with the insoluble fraction of thermally induced soy protein gels. Figure 6 and 7 support the hypothesis that intermolecular disulfide bonding influenced the subunit concentration of each fraction. Figure 6A and 6B show the protein banding profiles of control SPI, 3% SPI, and 8% SPI with soluble and insoluble fractions separated. The 8% SPI lost most of its soluble portion to the insoluble portion, probably due to a high degree of denaturation and a concentration capable of initiating aggregation. The control SPI profile is similar to the 3% SPI, except for a few heat sensitive disulfide bonded bands that are no longer present in the 3% SPI profile, such as the 11S acidic and basic disulfide bonded bands (Wolf 1993). Large molecular weight proteins were present in all of the fractions without β-ME and remained on top of the resolving gel. The large molecular weight proteins were reduced upon the addition of β-ME, indicating the presence of disulfide bonds in these polymers. Similar electrophoretic results were observed by Wang and Damodaran (1990). The fractions without β-ME contained large molecular weight polymers that streaked the gels, and were therefore filtered. The insoluble 8% SPI was too thick to filter, indicating that intermolecular disulfide bonds had most likely formed through protein contact during heating, resulting in large molecular weight polymers. The insoluble percentages for 8% SPI in figure 2.8b may be low because subunits were too large to migrate through the gel.

The SDS-PAGE revealed that the increase in aggregated and insoluble protein of the 8% SPI was a combination of the basic subunit, and the α', α, and β subunits, which disappeared from the soluble fraction (Figure 6A and 6B). The loss of these subunits to the insoluble fraction was also seen by Sorgentini and others (1995)

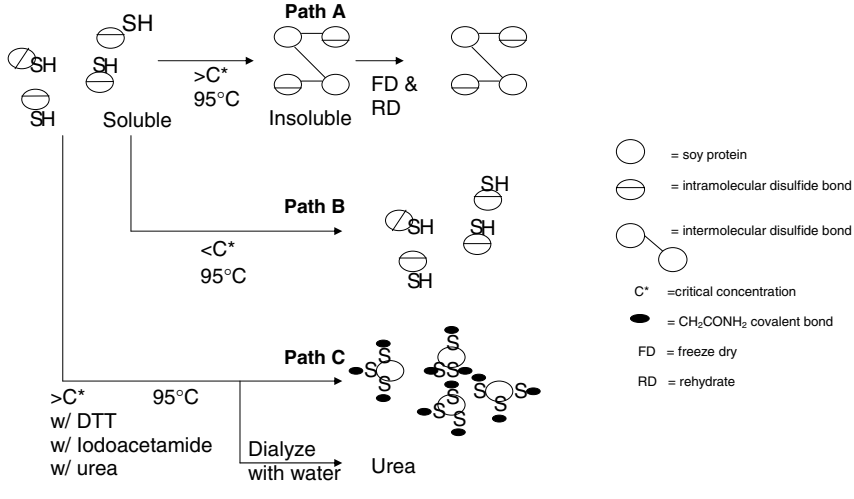

Figure 8—Proposed diagrammatic of disulfide interchange during heat induced gelation and effect of chemical modifying agents.

at high protein concentrations. Wolf (1970) observed the aggregation and loss of the basic subunit to the insoluble fraction during heating. Utsumi and others (1984) determined a decrease in the basic subunit and the β subunit in the soluble fraction at 0.5% protein concentration. The acidic subunit content increases in the soluble fraction when β-ME is present (Figure 7A), likely due to breaking of the acidic and basic disulfide bonds and noncovalent links during heating (Wolf 1993; Sorgentini and others 1995). The change of the basic subunit from the soluble to the insoluble fraction above the critical concentration would prevent acidic/basic interactions from reforming. When β-ME was not present, the acidic and basic groups were similar in amount for all except soluble 8% SPI, which had a high acidic subunit content (Figure 7B). The heat may break the acidic and basic unit in this case (Wolf 1993).

The progression of interactions producing the 8% SPI functionality is summarized in Figure 8. A critical concentration (C^*) is necessary to allow protein to contact and disulfide bonds to form until the protein is in a molecular weight range conducive to entanglements or hydrophobic interactions so that a gel network is formed (Path A). Heating below this concentration did not facilitate protein–protein interactions, and the protein irreversibly denatured without forming a gel network (Path B). The use of DTT and urea allowed the protein to unfold and disulfide bonds to be reduced, while iodoacetamide covalently linked to the reduced sulfhydryl groups. When these denaturants (DTT, urea, and iodoacetamide) were used, no viscosity increase was present, indicating a lack of protein–protein interaction (Path C). After urea was dialyzed out of the solution, hydrophobic interactions would be expected to occur during heating if they were an important part of the molecular interactions in soy protein gelation. The lack of any viscosity in the iodoacetamide reacted solution lends support to the idea that intermolecular disulfide bonds are the primary gelation mechanism of the cold-gelling soy protein isolate ingredient.

Conclusions

The sample preparation for the 8% SPI included a 2nd freeze drying step subsequent to heating for 3 h at 95 ± 3 °C to produce a convenient powdered ingredient. The temperature treatment of the 8% SPI eliminates the need for a heat step during product processing and allows the ingredient to be used in heat sensitive products. The 8% SPI has been observed to disperse quickly and show an immediate increase in viscosity in water at room temperature compared to the control SPI. The 8% SPI also maintains a stable viscosity through heating and cooling cycles. The 3% SPI did not show a viscosity increase compared to the control SPI and displayed significant changes during heating and cooling. The 3% SPI remained lower in viscosity than the control SPI and the 8% SPI after all samples were subjected to a thermal treatment to 90 °C at 8% protein. Differential scanning calorimetry showed that 3% SPI is denatured when thermally treated to 95 °C, indicating that without a high enough concentration, the proteins irreversibly denature to a form incapable of establishing the required interactions necessary for increased functionality, even upon reheating at a higher concentration.

The use of urea, DTT, and iodoacetamide decreased the viscosity of SPI dispersions. The effect of the denaturants on soy protein solutions supports the hypothesis that cold gelation functionality is produced through a combination of disulfide bonds, hydrophobic interactions, and hydrogen bonds. Disulfide interactions seem to be necessary for subsequent noncovalent interactions to occur. Therefore a concentration capable of inducing disulfide–sulfhydryl contact is required initially.

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